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Tests for Bactericidal Effects of Antimicrobial Agents: Technical Performance and Clinical Relevance

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INTRODUCTION

Antimicrobial susceptibility testing is an important task for the diagnostic microbiology laboratory because the results are used by practicing physicians to determine treatment regimens for patients with infections. The microbiology laboratory's approach to susceptibility testing, as it relates to the management of infectious diseases, is the topic of many reports (17, 24, 37, 38, 47, 49, 51, 55, 60, 73, 74, 94, 111). The questions facing clinical microbiologists are how to best perform such in vitro tests and which ones are most useful for guiding therapy for the infected patient (12, 98). Many methods have been proposed since work measuring the susceptibility of microorganisms to antimicrobial agents began in the early 1920s (61). Selected tests have been used widely in the practice of clinical microbiology (61, 74). Recommended methods for performing both routine and specialized testing have now been published (75–77).

Lorian and Burns (64), who recently discussed the usefulness of laboratory susceptibility testing, found a high correlation between the results of routine, qualitative (susceptibility versus resistance) in vitro susceptibility tests and the outcome of therapy in a retrospective analysis of 298 patient records. However, the positive relationship between the results of even routine quantitative (MIC) in vitro testing and therapeutic outcome has not been uniformly observed. Linder and Fass reported no correlation between laboratory test results and a favorable response to therapy for 21 patients with bacteremia caused by gram-negative organisms who were treated with aminoglycosides (63). The only factor that correlated with a favorable outcome was coadministration of an active β-lactam agent. More recently, Hilf et al. (45) reported a comparable outcome in a larger series of 200 patients who were bacteremic with *Pseudomonas aeruginosa*. They failed to demonstrate a direct correlation between quantitative in vitro susceptibility test results and therapeutic outcome. A current review by Korvick and Yu highlights the problem of interpreting in vitro susceptibility test results for this pathogen (60). Similar results were reported by Van der Auwera et al. for immunocompromised patients infected with gram-positive bacteria and treated with vancomycin or teicoplanin (113). On the other hand, recognition of bacterial isolates resistant to antimicrobial agents is critical and a prime concern of the clinical laboratory. Properly performed routine bacteriostatic tests, such as disk diffusion and MIC testing, can detect resistance resulting from many mechanisms. Clinicians must be notified of such occurrences to avoid the use of potentially ineffective regimens (98).

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For most in vitro susceptibility testing, the methods test an agent's ability to inhibit or suppress bacterial growth (bacteriostatic tests), but occasionally a special assessment of bactericidal activity is sought. Bactericidal testing has multiplied laboratory problems in susceptibility testing because bactericidal tests are more labor intensive and are not well standardized. The medium used for testing, the growth phase of the inoculum, the incubation time and temperature, whether the tubes are stationary or shaken, the presence of serum proteins in the growth medium, the size (volume) of the incubation vessel, the volume of subculture, antibiotic inactivation at subculture, and even the conditions under which the bacterial isolate is stored before testing can alter the test results, often in unpredictable ways. The problem of reproducibility in bactericidal testing was again demonstrated in 1990, when James reported that the test results for both macrodilution and microdilution test procedures are not reproducible (50). When the activity of penicillin was tested against 28 strains of viridans streptococci, routine MIC test results were consistent within 1 doubling dilution; however, the 95% confidence limit for macrodilution MBC tests was ±3.47 doubling dilutions, and for the microdilution MBC tests, it was ±4.24 doubling dilutions. Such a range between duplicate test results (seven- to eightfold) made the results uninterpretable.

A particularly difficult form of resistance to detect (from the viewpoint of the clinical laboratory) is antibiotic tolerance, or the inability of normally bactericidal agents to exert a lethal effect (105, 112). This phenomenon has been linked to a slow clinical response to therapy, particularly in the treatment of staphylococcal endocarditis (62). Tests for bactericidal activity are often considered important for bacterial endocarditis. Tolerance is generally found in association with cell wall-active antibiotics and has been defined as "a reduction in the rate of antibiotic-induced killing of a whole bacterial population, relative to some standard culture" (112). An obvious problem with this definition is the absence of an accepted standard for comparison. In vitro, tolerance has been demonstrated clearly for bacteria defective in antibiotic-induced autolytic activity (44, 112), but phenotypic tolerance also occurs in cells deprived of nutrients, which typically multiply slowly or do not grow. The common method for detecting tolerance is the MBC test, described in a later section. The test organism is defined as tolerant if the ratio of the MBC to the MIC is ≥32 (96, 105). This particular approach to detecting tolerance is highly subject to the technical variables of the MBC test. Most authorities recommend the use of complex time-kill data (defined in a later section), a technique not well suited to the clinical laboratory, as the method of choice for laboratory detection of tolerance (44, 105).

Other clinical instances in which tests for bactericidal activity are recommended by some authorities include bacterial endocarditis (17, 47, 94), sepsis in the immunocompromised patient (17, 94), infections in those unable to mount an immune response (23, 47, 55), osteomyelitis (47, 111), chronically infected implants (47), other types of chronic infections (47), and prediction (or monitoring) of therapeutic efficacy (as with a serum bactericidal titer [SBT] test) in infectious diseases for which there are no therapeutic guidelines (27). MacLowry has recently focused on the problems associated with bactericidal testing, with special reference to the SBT test (66). While he was specifically commenting on the use of the SBT test, many of his concerns are directly applicable to bactericidal testing in general. His recommendation for evaluating the reproducibility and clinical relevance of standardized methods before using them in clinical laboratories is sound and applicable to all bactericidal testing.

Although in vitro bactericidal tests have been performed for many years, questions remain about the best methods to use and the clinical relevance of the results (6). Indeed, even the terminology frequently associated with such tests is often confusing. The purpose of this review is to (i) describe methods that have been suggested for bactericidal tests in the clinical laboratory, (ii) evaluate the clinical data for the likely role of bactericidal testing in the treatment of infected patients, and (iii) recommend when in vitro bactericidal testing can be most useful as well as which test method(s) is most likely to give reproducible results.

Terms Associated with Bactericidal Testing

Many terms are associated with the bactericidal testing of antimicrobial agents in the laboratory. The most frequently used terms are defined here.

MIC. The MIC is the lowest concentration of an antimicrobial agent that inhibits growth, as determined visually after a standard incubation period (usually 18 to 24 h).

MBC and minimum lethal concentration. The MBC is the lowest concentration of an antibacterial agent that causes at least a 3 $\log_{10}$ reduction in the number of surviving cells (compared with the initial, preincubation concentration) after incubation (usually 18 to 24 h). When the term minimum lethal concentration is used, the test referred to can be directed against any microbe, not just bacteria (e.g., fungi and viruses).

Serum inhibitory titer. The serum inhibitory titer is the highest dilution (or titer) of a serum sample taken from a patient receiving antimicrobial therapy that inhibits visible growth after incubation (usually 18 to 24 h). The test is typically run against the organism(s) recovered from an infected site of the patient being evaluated. This test was described by Schlichter and MacLean in 1947 (100).

SBT. The serum bactericidal titer (SBT) is the highest dilution (titer) of a serum sample taken from a patient receiving antimicrobial therapy that causes at least a 3 $\log_{10}$ reduction in the number of surviving cells (compared with the initial inoculum) after incubation (usually 18 to 24 h). This test has also been referred to as the serum dilution test and (incorrectly) as the Schlichter test. When the term serum dilution test is used, the test referred to can be directed against any microbe, not just bacteria.

Paradoxical effect (*Eagle phenomenon*). This is the phenomenon in which an unexplained, increasing number of surviving cells (indicating decreasing bactericidal activity) are seen as the antimicrobial agent concentration increases above the MBC (46).

Persisters. Persisters are small numbers of cells (usually less than 0.1 or 0.01% of the initial inoculum) that survive the lethal effect of antimicrobial agents at concentrations that exceed the MBC. These "persisting" cells have the same susceptibility as the original strain.

Skip tubes. This is the phenomenon in which one or more tubes in a series of broth dilutions appear to be free of bacterial growth (or contain ≥3 $\log_{10}$ fewer bacteria than the initial inoculum), whereas one or more tubes containing higher concentrations of antimicrobial agent contain <3 $\log_{10}$ fewer bacteria than the initial inoculum; i.e., individual tubes containing a drug concentration below the MBC are found to be sterile.

Tolerance. Tolerance is the phenomenon in which nor-

mally bactericidal agents (e.g., β-lactams and vancomycin) appear to have reduced or absent bactericidal activity against selected bacterial strains. The mechanism is often thought to be impaired bacterial autolytic enzyme activity, although other mechanisms likely exist. Many authors also include an MBC/MIC ratio of ≥32 as part of the definition of tolerance.

Killing curve (time-kill study). This is a technique similar to that used for the MBC test except that only a single antibiotic concentration is typically studied (usually near the mean achievable level in blood) and subcultures to antibiotic-free agar are done at multiple times during a 24-h incubation period. The test permits the actual rate at which the number of viable bacteria decreases from that in the original inoculum, and thus the bactericidal rate, to be determined.

Checkerboard (synergy) test. This test is used for combinations of two antimicrobial agents to determine whether the combination is more or less active than either drug given alone. Doubling dilutions of each agent are prepared, one increasing with the vertical axis and the other with the horizontal axis, with each tube in the entire set (often containing up to 144 tubes in a 12-by-12 configuration) containing a different concentration of each of the two antimicrobial agents. The appearance of bacteriostatic or bactericidal activity is typically determined for each tube in the set, with the result indicating the optimal concentrations of each drug in combination for inhibitory and bactericidal effects.

IN VITRO METHODS FOR DETERMINING BACTERICIDAL ACTIVITY

Factors Influencing Test Outcome

The College of American Pathologists has been concerned with the accuracy of routine (bacteriostatic) antimicrobial agent susceptibility tests for many years and has reported improved performance during the period from 1972 to 1983 (52). In the 1981 to 1983 College of American Pathologists microbiology surveys, Jones and Edson (52) reported 95% or better accuracy by test participants for both the disk diffusion and dilution (MIC) assays. However, this good performance was not reflected in the survey done on the serum dilution test (SBT) as a measure of special tests for bactericidal activity (66). MacLowry reported that the range of titers, from 1:2 to 1:512 (for both inhibitory and bactericidal titers), made the results of these tests virtually uninterpretable (66). While improved assessment of clinical value is important for any test, Greenwood has suggested that the multiple factors influencing dilution susceptibility testing results have the potential to make them "inherently irreproducible" (38). This is especially true if a variety of different or nonstandard test methods are used. Therefore, the issue of reproducibility in MBC testing must be addressed even before any clinical evaluation can proceed.

Several reports (44, 105, 112) have described well the technical difficulties of determining tolerance in clinical laboratories as well as the variables that affect the serum dilution (SBT) test (120). These difficulties must be dealt with before bactericidal determinations (including MBC) can be made reproducibly. These four reports outline the major technical factors influencing the outcome of such tests, including growth phase of the inoculum, adherence of bacterial cells to the walls of the test vessel, incubation times, medium content and pH, and antibiotic carryover. The many variables that influence the outcome of the MBC test include the broth used (59, 67, 78, 85, 99, 104, 114, 120), the use of cation supplements (41, 92, 107, 120), whether the culture is mixed or otherwise agitated during incubation (48, 104, 105, 108, 120), the growth phase of the inoculum (7, 39, 48, 57, 70, 104, 105, 108, 114, 120), the pH of the medium (34, 105, 114, 120), the use of serum (binding) proteins (92, 120), the presence of complement (21, 34, 120), the elimination of antibiotic carryover during subculture (8, 25, 35, 47, 93, 103, 104, 119, 122), the final density of the bacterial inoculum (1, 99, 120), the endpoint cutoff (2, 4, 22, 75, 83, 107, 119), the incubation time (41, 80, 105) and temperature (65), the storage conditions and time in storage before testing (70), the depletion of nutrients during incubation (13), the volume of subculture used (104), and the temperature of the medium (121). With all these variables, it is not surprising that there are at least 13 reports citing major concerns with reproducibility (1, 34, 41, 78, 80, 85, 99, 104, 105, 108, 120, 121, 123), indicating that most clinical laboratories have a major problem obtaining accurate bactericidal test data.

Standardization of MBC Tests

Because of the marked variations in the methods used to perform tests that assess the bactericidal activity of antimicrobial agents (88), two methods for performing the MBC test have been proposed. The basic characteristics of these proposed standard methods are shown in Table 1. Each of the components is important, and many of the variables listed above are addressed.

Medium. Currently, most authorities recommend Mueller-Hinton broth, either alone or supplemented with serum, at a final pH of 7.2 to 7.4 (75, 102, 106a, 114), as the MBC test medium. In early studies, Mueller-Hinton broth gave MBCs that were in close agreement with the MICs of semisynthetic, antistaphylococcal penicillins against *Staphylococcus aureus*, which is likely the initial basis for the selection of this test medium (67). However, recent work has found that the effect of the medium is variable and unpredictable in tests of *S. aureus* (78, 85). Mueller-Hinton broth more closely resembles plasma and serum in pH, Na^+, K^+, and Cl^- concentrations, and osmolality than do many other broth media and was selected by the National Committee for Clinical Laboratory Standards (NCCLS) as the medium of choice for this test (75). Supplements must be added for certain tests, for example, to facilitate the growth of fastidious organisms such as *Haemophilus* species and some streptococci. Additional divalent cations are needed when testing aminoglycosides, and sodium chloride should be added (2%) for testing penicillinase-resistant penicillins against *S. aureus*. However, Stratton and Cooksey recently recommended against adding sodium chloride when testing methicillin-resistant *S. aureus*, as they found that salt strongly inhibited killing of these strains by β-lactam agents (106a).

We prefer not to add serum to our MBC test broth because it is yet one more uncontrolled variable in the method. Serum has long been known to modify the activity of antimicrobial agents. Antibacterial activity can be enhanced by bactericidal factors in serum (110) or inhibited by binding to serum proteins (92). In tests without serum, often done with commercial microtiter trays for MIC testing without serum, the results are read as the concentration of free (non-protein-bound) antimicrobial agent needed to kill (≥3 $\log_{10}$ reduction in bacterial density over 24 h) the test organism in the absence of any aid from the host immune system. The concentration of free drug available at most

TABLE 1. Proposed reference methods for MBC tests

Test component	Recommendation	
	NCCLS[a]	Stratton and Cooksey[b]
Broth medium	Mueller-Hinton, with appropriate supplements	Same (no NaCl for methicillin-resistant *S. aureus*)
pH	7.2–7.4	None
Serum addition	Optional	None
Inoculum	Growing cells from 4–5 colonies	Growing cells from 20–30 colonies
Final inoculum density	5×10^5 CFU/ml	Same
Inoculation	Add below broth surface without shaking or agitation	Same
Incubation temp and time	35°C, 24 h (agitate macrodilution tubes at 20 h)	Same
Subculture	Duplicate 0.01-ml samples on agar plate; 0.01- to 0.1-ml sample for microdilution	Single 0.01- to 0.1-ml sample
Endpoint	99.9% reduction of initial inoculum	Same
Quality control procedures	Described	None
Control strains included in results	Yes	No
Micro- or macrodilution preferred	Neither preferred	Microdilution

[a] From reference 75.
[b] From reference 106a.

body sites can be estimated adequately from published reports (32, 83, 84). Knowing these levels can be useful when interpreting MIC or MBC test results. The report by Gerding et al. (32) contains tables indicating the expected concentrations of most antibiotics at various extravascular sites in humans. The reports by Peterson and Gerding (83, 84) give techniques for predicting antimicrobial agent concentrations at extravascular sites, based on the established serum pharmacokinetics and protein binding of the drug(s) of interest.

Inoculum. Both the size and preparation of the inoculum are important factors and need to be carefully controlled. The standard inoculum concentration chosen by the NCCLS is 5×10^5 CFU/ml of broth medium (75). Changes in inoculum density as well as the absolute number (rather than concentration) of total organisms present in the test system (a potentially important 10-fold difference between the macro- and microdilution test methods) can affect the outcome of both MIC and MBC tests. A 5×10^5 CFU/ml inoculum provides an acceptable challenge dose for assessing the biological activity of antimicrobial agents and is large enough to provide statistically satisfactory data for determining an MBC endpoint. If the inoculum is too small, significant bacterial resistance may not be detected.

To prepare the inoculum, at least four to five isolated colonies ≤24-h old are inoculated into 4 to 5 ml of a suitable broth and incubated for 3 to 5 h, i.e., grown to visible turbidity (mid-logarithmic phase). The growth is visually adjusted to match the turbidity of a 0.5 McFarland standard and then finally diluted so that the concentration of bacteria after inoculation into either macrodilution test tubes or microdilution wells is 5×10^5 CFU/ml of broth. The proposed NCCLS procedure recommends an inoculum of 5×10^6 CFU/ml for anaerobic microorganisms (75). The initial organism density in the tubes or wells is critical in the MBC test and must be determined accurately before the final MBC endpoint analysis can be made. Even when the inoculum density is standardized, the total number of organisms per tube or well depends on the volume of the incubation vessel (macro- versus microdilution), and this absolute number of organisms can affect the outcome. A large enough inoculum is particularly important for detecting mutational resistance among isolates with low mutation frequencies (e.g., aminoglycosides or fluoroquinolones tested against many gram-negative bacilli) and when resistance is due to the production of inactivating enzymes, such as β-lactamases.

Organisms in the log phase of growth are used for this test because many antimicrobial agents (particularly those that interfere with cell wall synthesis, such as β-lactam compounds and vancomycin) require active bacterial growth to exert lethal activity. While the growth phase may not have a significant effect on MIC tests, as demonstrated by Barry et al. (7) and Kim and Anthony (56), most investigators (48, 56, 70, 75, 108, 114) have confirmed the importance of using log-phase growth for the inoculum for MBC tests. When stationary-phase growth is used, the number of surviving or "persisting" organisms tends to increase after 24 h of incubation, and therefore the MBC result may be artificially high. Similarly, the use of even late-logarithmic-phase growth exaggerates the paradoxical effect (108).

The manner of adding the starting bacterial inoculum is critical and can markedly influence the outcome of MBC tests (39, 104, 105, 108). Inoculation must be done so that any splashing onto the insides of the test vessel is avoided. If splashing occurs, bacteria on the vessel walls may not come into contact with the antimicrobial agent(s) being tested and thus appear to be falsely resistant. The initial inoculum is added by gently releasing it (in a total volume of ≤0.1 ml) beneath the surface of the antimicrobial agent-containing medium. This step is most easily accomplished by the microdilution test procedure because the multipoint inoculator places the inoculum directly into the center of the medium-containing wells.

Antimicrobial agents. Antimicrobial agents of known potency should be used when preparing dilutions for testing. If fresh stock solutions are not prepared for each determination, they need to be stored so as to preserve antimicrobial activity. Storage at −70°C is adequate for most stock solutions of antimicrobial agents. The potency must be determined when performing MBC tests, either by direct drug

assay or by quality control testing with microorganisms of known susceptibility.

Incubation. Incubation conditions need to be tightly controlled because they too can have a major impact on the test outcome. Incubation should be done at 35°C. Altering the temperature can affect the results because higher incubation temperatures augment the effects of some antimicrobial agents (65). Microdilution trays should not be stacked too high (generally no higher than four) to ensure that all trays are exposed to 35°C for similar periods of time. The NCCLS proposed standard recommends that macrodilution tubes be agitated gently after 20 h, reincubated for 4 h, and then agitated again before sampling to ensure complete mixing (75). Taylor et al. evaluated the effect of inoculum on the outcome of macrodilution tests (108). They found that gentle agitation at 20 h was useful when a large-volume inoculum (1.0 ml) was used; however, it was not needed when the recommended ≤0.1-ml inoculum volume was used (108). We (41) and Ishida et al. (48) have found it preferable to always use the smaller inoculum (≤0.1 ml) and not to disturb the sample until the time of actual subculture sampling.

When the MBC test is performed by the suggested reference methods, all cultures for MBC determinations should be sampled after 24 h of incubation. However, other reports suggest that under certain circumstances, an additional 24-h incubation period may be useful for macrodilution broth tests (41, 80, 81). Pelletier reported lower MBCs for several antimicrobial agents against *S. aureus* after 48 h than after 24 h (80). He also found better agreement for many of the replicate tests when subculture was repeated after 48 h of incubation. We found that a 48-h incubation eliminated much of the "skipping" problem (i.e., one or more macrodilution tubes with a bactericidal [$\geq 10^3$-fold] reduction in bacterial counts in the midst of tubes containing higher growth levels) in tubes that showed skips after 24 h of incubation (41). When performing the MBC test by the macrodilution method, we believe it is imperative to run the test in duplicate and to incubate all tubes for an additional 24 h after the initial sampling. In this way, they can be resampled at 48 h if the duplicate 24-h samples yield discrepant results (>1 doubling dilution difference) or if one of the test dilution sets shows skips.

Subculture. The MBC test is quantitated by subculture of all nonturbid wells or tubes at 24 h and, if needed, at 48 h. The sample for quantitative analysis needs to be large enough to provide sufficient bacterial cells for an accurate endpoint determination, but not so large as to pose difficulty with antibiotic carryover, giving a falsely low MBC (4, 8, 48, 95). The proposed standard recommends a subculture volume of 0.01 ml for macrodilution and 0.01 to 0.1 ml for microdilution tests (75). A 0.01-ml sample will provide ≤5 colonies when an initial inoculum of 5×10^5 CFU/ml is used, and therefore, we believe that both macro- and microdilution subcultures should contain a 0.1-ml sample. Barry and Lasner found increasing problems with drug carryover when subculture sample sizes were greater than 0.01 ml, but their testing was done by spotting (without spreading) the subculture inoculum on the agar plate surface (8).

Before sampling, macrodilution tubes are gently mixed, and the sample is removed by calibrated pipette. For microdilution panels, each well is gently stirred with the pipette tip before the contents are aspirated. Each sample is then placed on a single antibiotic-free agar plate suitable for the growth of the microbe being tested, and the plates are incubated for a full 24 h (sometimes longer for slowly growing microbes) at 35°C. Proper dispersion of the subculture sample on the agar plate is important, especially for samples containing higher concentrations of antimicrobial agents (25). The method used must prevent the antibiotic contained in the transferred broth from simply suppressing the growth of viable bacteria contained in the subculture sample. Various techniques are available for inactivating antimicrobial agents in agar (35, 73, 82), including the use of β-lactamase for β-lactam agents. However, we have found that the easiest and most reliable technique for avoiding carryover antimicrobial agent interference is to place the sample onto the agar plate in a single streak down the center, allow the broth to be absorbed into the agar until the plate surface appears dry, and then spread the inoculum over the plate with a sterile bent glass rod (104). We have used this method successfully for both gram-positive cocci (28, 87, 104) and gram-negative bacilli (5, 27, 33, 72, 86).

MBC endpoint calculation. The MBC result should be determined by one of two procedures. One method is that of Pearson et al. (79), which uses the number of viable colonies growing on the 24-h or 48-h subculture plate and the number of viable colonies in the starting inoculum. They used Poisson probabilities to calculate the endpoint rejection values (cutoff number of colonies for 99.9% killing), based on a defined initial inoculum ranging from 10^5 to 10^7 CFU/ml and a subculture sample size of 0.01 ml. They also report on the sensitivity and specificity of the MBC test results derived from their calculated rejection limits. If a sample size of 0.1 ml is used, the values obtained by Pearson et al. must be recalculated, but the sensitivity and specificity of the test are better with the 0.1-ml sample size, especially when lower initial inoculum densities are used. We (41) have also found that the formula $n + 2\sqrt{n}$, suggested by Anhalt et al. (2), for determining the MBC cutoff point is a simple, understandable, and useful alternative to the calculations of Pearson et al. This calculation, in which n is 0.1% of the initial inoculum and $n + 2\sqrt{n}$ is the corrected MBC cutoff point, provides a quantitative endpoint that includes the 95% confidence limit for the determination of 99.9% killing. The first well or tube whose 24- or 48-h subculture contains the number of colonies below the cutoff point calculated to represent a ≥99.9% reduction in bacterial cells is considered to contain the MBC of the drug being tested.

The reason for the selection of a 3 $\log_{10}$ reduction from the starting bacterial density as the MBC cutoff point is somewhat unclear (104). This value was apparently first chosen because it represented total elimination of bacteria from a starting inoculum of 10^3 CFU/ml (22). Therefore, if Eagle and Musselman's approach is used for an endpoint determination, the required MBC cutoff point for an initial inoculum density of 10^5 CFU/ml should be a reduction in bacterial density of 5 $\log_{10}$. However, the 3 $\log_{10}$ or 99.9% reduction as the cutoff point for determining the MBC is the currently accepted standard, and this rather arbitrary endpoint has been used as a reference value for many years. The 3 $\log_{10}$ reduction from initial inoculum density is part of the definition of most tests for bactericidal action, and it is a historical reference point for the comparison of any new test methods that assess the bactericidal action of antimicrobial agents.

The problem of inconsistent growth or skips in tubes or wells makes interpretation of test results difficult. If a single test is performed and any skips are noted in the 24-h subculture, a 48-h subculture must be performed. The 48-h results are then used as the basis for the test interpretation (assuming there are no skip tubes at 48 h). If tests are performed in duplicate and one of the two sets demonstrates a skip at 24 h, then the other set can be used for the result

TABLE 2. Proposed changes to NCCLS reference method for MBC tests

Test component	NCCLS method[a]	Suggested modification
Broth medium	Mueller-Hinton with appropriate supplements	None
pH	7.2–7.4	None
Serum addition	Optional	Not recommended
Inoculum	Growing cells from 4–5 colonies	None
Final inoculum density	5×10^5 CFU/ml	None
Inoculation	Add below broth surface without shaking or agitation	None
Incubation temp and time	35°C, 24 h (agitate macrodilution tubes at 20 h)	No agitation until sampling
Subculture	Duplicate 0.01-ml samples on agar plate; 0.01- to 0.1-ml sample for microdilution	Single 0.1-ml sample; run test in duplicate, do 48-h subculture for discrepancies or skips
Endpoint	99.9% reduction of initial inoculum	$n + 2\sqrt{n}$ calculation
Quality control procedures	Described	None
Micro- or macrodilution preferred	Neither preferred	Microdilution

[a] From reference 75.

determination. However, in this case, we prefer that 48-h subcultures be done on both sets, with a final interpretation based on those results. If both sets show skips at 24 h, then 48-h subcultures should be performed on both sets and any set whose dilution series has test results free of skip tubes should be used for determination of the MBC. We have found that this method and use of the formula $n + 2\sqrt{n}$ to calculate the cutoff endpoint (2) provide the most consistent, reproducible results for MBC testing (41). Other unusual test result patterns, such as discrepant results between paired tests at both 24 and 48 h, bacterial growth at high antibiotic concentrations with none at lower concentrations, failure of growth in the control well or tube, and growth in the sterility control well or tube, require that the entire test be repeated.

The reason for the skip phenomenon, which confuses the interpretation of MBC test results, is unclear. Some postulate that it is caused by persisters, metabolically inactive cells that survive the lethal action of antimicrobial agents (40, 42). When placed on antibiotic-free solid agar, they regrow, and thus the antimicrobial agent appears to lack bactericidal activity in random wells or tubes. The paradoxical effect, a phenomenon described before 1950, is seen when increasing concentrations of an antimicrobial agent result in diminished bactericidal activity against the organism under study (22, 46, 91). We have been unable to correlate this effect with the skip phenomenon (41). Holm et al. recently reported findings that appear to link it to a mechanism similar to that responsible for tolerance (46).

Quality control. Quality control is vital to bactericidal testing. Strains that are well characterized and useful for quality control testing can be purchased from the American Type Culture Collection, and the NCCLS has recommended four such strains: *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 (75, 92). Whatever strains are chosen, they should (i) give reproducible results; (ii) give results in the mid-range of the antimicrobial agent concentrations being tested; and (iii) be stored so that they will be available for a number of years. The quality control strain(s) should be included each time an MBC test is performed. A growth control must also be included for each organism, and it can be subcultured at 24 h as a purity check. Finally, an uninoculated sterility control should be included as a control for the sterility of the test medium.

Proposed MBC Test Procedure

Our suggested modifications to the proposed NCCLS test procedure (75) are summarized in Table 2. We avoid the use of a serum additive because it introduces a major, uncontrolled variable to the test, and the direct effects of serum on antimicrobial agent performance due to protein binding during patient treatment can be estimated easily. Because of the poor reproducibility of the MBC test, it should always be run in duplicate. When the macrodilution method is used, the subculture should be repeated after 48 h of incubation if the results at 24 h show skips or are discrepant. Also, we do not recommend any agitation before sampling. As noted earlier, Taylor et al. (108) demonstrated that early agitation was helpful only with a large initial inoculum volume of 1.0 ml, and not when ≤0.1 ml was used. Early agitation may remove viable bacterial cells from continued exposure to the antimicrobial agent (48, 108). A sample size of 0.1 ml for the subculture gives sufficient precision to the endpoint determination used in the evaluation of the MBC result. Finally, we prefer to use the microdilution procedure because the results appear to be more reproducible than those obtained with the macrodilution method. When the microdilution method is used, however, there is no opportunity to extend the initial incubation time from 24 to 48 h because the entire inoculum is sampled during the subculture step.

OTHER METHODS USED TO TEST BACTERICIDAL ACTIVITY

Bactericidal Rate Analysis and Tests for the Effect of Antimicrobial Agents in Combination

Measurement of the rate of bactericidal activity, or time-kill analysis, has been considered a clinically relevant replacement for or adjunct to MBC testing. Drake et al. have reported that an 8-h time-kill test is useful for predicting cure in experimental endocarditis (19). We have found that a similar 6-h test predicts in vivo success for selected antimicrobial agent-bacteria combinations tested in experimental (animal model) infections (72, 87). However, as a comparison to more traditional 24-h tests, we have evaluated 23 strains of *S. aureus* against vancomycin, methicillin, cephalothin, and gentamicin in microdilution trays for 4 and 24 h (104). Except for gentamicin (a representative of a class of

agents that rapidly express bactericidal action), the results at the two time points (4 and 24 h) showed little correlation. It appears that an incubation period of more than 4 h is required if a shortened time-kill test is to be useful for evaluating most antimicrobial agent-bacteria interactions. Bayer and Morrison have commented on the disparity between the results of time-kill tests and the results of the often-used checkerboard test when they are used to evaluate synergistic bactericidal interactions with combinations of antimicrobial agents (9). Recently, Briceland et al. proposed that time-kill tests (particularly those involving the direct assay of a patient's serum sample) may improve the clinical utility of in vitro determinations of bactericidal activity (11). However, we are unaware of any prospective, controlled clinical comparison of any of these newer methods.

A tentative method for time-kill testing is included in the NCCLS document M26-P (75). The same critical factors that affect the outcome of the MBC test affect the performance of this test. Additionally, Woolfrey and Lally have reported that when bacteria are enumerated by a pour-plate method instead of by broth or saline dilution followed by plating on solid agar, the temperature of the molten agar used for sample quantitation can affect the test results (121). In this study, the authors found that the 50°C temperature of molten agar killed some strains of *S. aureus*. Also, whenever time-kill tests are compared with checkerboard tests for assessing the activity of antimicrobial agents in combination, the calculation used to determine synergy in checkerboard tests should be a fractional bactericidal index (ΣFBC) (43): ΣFBC = (MBC of antibiotic *a* in combination/MBC of antibiotic *a* alone) + (MBC of antibiotic *b* in combination/MBC of antibiotic *b* alone). A calculated ΣFBC of ≤0.5 indicates synergy, a ΣFBC of >0.5 to <4.0 indicates indifference, and a ΣFBC of ≥4.0 indicates antagonism. We are unaware of any prospective clinical evaluations of these types of bactericidal tests, and until those data are available, we believe that time-kill testing results are not appropriate for direct, individualized application to the therapy of infectious diseases.

Bactericidal Testing on Solid Agar

Bactericidal analysis of antimicrobial activity in solid rather than liquid medium has also been proposed (16, 50, 68, 69, 82, 122). Most techniques require inactivation of the antimicrobial agent under evaluation after the initial 18- to 24-h incubation period, and the majority of these studies have involved inactivation of β-lactam agents with a β-lactamase (50, 68, 69, 82). Fernandes et al. (30) and Kim and Anthony (57) have described a membrane-based testing method by which microorganisms can be transferred to fresh antibiotic-free agar after the initial incubation; workers can then test for bactericidal activity without having to inactivate the antimicrobial agent present in the initial test agar. This approach is potentially applicable to a broader range of antimicrobial agents than are agar-based tests that require antimicrobial agent inactivation. However, James recently reported that the membrane test is nonreproducible (45). Less is known about the variables that influence the performance of any of these newer tests, including the recently marketed E-test, than about those that influence the more traditional MBC test (96a), and none of these tests has been evaluated prospectively in a clinical investigation.

Disk Elution Method

A modification of the broth macrodilution method in which routine antibiotic susceptibility testing disks are used to determine both MICs and MBCs was reported by Wilson et al. (118). The authors claimed that the reproducibility of their method was good and that it was comparable to the NCCLS reference method (77) for susceptibility testing. However, they used a suboptimal subculture volume of 0.01 ml and found that only 70 to 84% of the expected oxacillin concentration was eluted from the antibiotic disk during a 1-h elution period in broth (118).

CLINICAL RELEVANCE OF BACTERICIDAL TESTING

Animal Models

Animal models of infection are one way to study the usefulness of in vitro tests of bactericidal activity because they provide a more controlled setting than infected patients for the correlation of in vitro test results with in vivo outcome (25a). Attempts have been made to validate the use of MBC testing as a predictor of therapeutic outcome by using animal models of infection. We have reported several studies in a rabbit model that in many respects closely simulates the in vitro test conditions used for MBC testing. Such a model should provide the optimal setting for the comparison of in vitro and in vivo interactions. The model uses implanted Visking semipermeable membranes, which permit free diffusion of molecules with molecular weights of ≤15,000. Antimicrobial agents therefore diffuse freely, while all cells and other host immune factors are excluded. Bacterial metabolic waste products diffuse from the area and are carried away by the animal's blood supply outside the membrane chamber. The in vitro tests evaluated include standard and high-inoculum MIC and MBC tests, killing curve tests, incubation of MIC and MBC test cultures anaerobically as well as aerobically, and checkerboard testing for synergy when combinations of agents were used. Even in this highly controlled in vivo environment, with few variables contributed by any host immune factors and numerous in vitro test methods evaluated comparatively, it was not possible to precisely predict the in vivo outcome from quantitative MBC data (5, 27, 28, 33, 72, 86, 87). Similar results were recently reported by Widmer et al. in a model of foreign-body infections (117). They found that for the results to correlate directly with the results of therapy, the in vitro test conditions needed to mimic the in vivo infection site conditions closely. Paralleling this observation, Chuard et al. found that *S. aureus* recovered from a foreign body was much less susceptible to the bactericidal action of antibiotics than were the parent strains of the isolates tested (14). However, Fantin et al. have used a thigh infection model in mice and reported good correlation between MIC and MBC test results and therapeutic outcome when testing tobramycin, pefloxacin, ceftazidime, and imipenem against gram-negative bacilli (26).

These animal model studies illustrate the problem described by Zak et al. (124); that is, quantitative correlation of the results of in vitro tests of bacterial susceptibility with in vivo (animal) response to antimicrobial therapy is difficult even when highly standardized and reproducible tests based on inhibitory activity are used. Their review concluded that "for the time being, it seems unrealistic to expect that in vitro tests could be developed that would make it possible to [quantitatively] predict the efficacy of any antibiotic against any specific infection in vivo" (124).

Animal studies have also demonstrated another difficulty in the clinical interpretation of MBC test results, that of the individual infected with a tolerant microorganism. If tolerance is loosely defined as inhibition but not killing by the usually effective concentrations of typically bactericidal agents, the reports based on retrospective studies in humans do not show any direct relationship between in vitro recognition of this phenomenon and therapeutic outcome (18, 54, 90). Experimental evaluations of the tolerance phenomenon in animal models have also provided conflicting results. In an early study, Goldman and Petersdorf (36) made two important observations: (i) they showed that by changing the broth medium from Trypticase soy to Mueller-Hinton broth, they could convert a tolerant *S. aureus* strain to a nontolerant isolate, illustrating the difficulty faced by most clinical laboratories with in vitro testing for phenotypic expression of tolerance (even the medium selected will markedly affect test outcome); and (ii) they demonstrated that both tolerant and nontolerant staphylococci respond in the same way to methicillin given prophylactically or therapeutically, showing the difficulty of correlating any in vitro results of tests for tolerance (even if performed in a "standard" manner) with therapeutic outcome. Subsequently, however, Brennan and Durack (10) and Kim and Bayer (58) have reported data supporting an in vivo effect of in vitro-determined bacterial tolerance in experimental streptococcal endocarditis. A longer time was needed to eradicate tolerant than nontolerant strains of both *Streptococcus sanguis* and *Enterococcus faecalis* during chemotherapy with penicillin. Voorn et al. also found an animal model for *S. aureus* endocarditis which supported the correlation of in vitro testing showing tolerance and diminished therapeutic efficacy in vivo (115).

Animal models also have been used to develop and evaluate new tests of the bactericidal activity of antibiotics in attempts to improve current conventional tests. Using the model of bacterial endocarditis, Drake et al. have shown that a brief (8-h) killing-rate analysis is superior to a 24-h MBC test in predicting the efficacy of combination therapy for *S. aureus* endocarditis (19). However, Fass found no test useful in predicting outcome in experimental endocarditis (29), and as far as we know, the use of newer tests for monitoring antimicrobial agent treatment of infections in humans has not been systematically studied.

In summary, animal models have been used extensively to compare the findings of quantitative tests of in vitro susceptibility (particularly MBC and SBT) with therapeutic outcome, with complex and conflicting results. It is clear from these studies that there is no simple relationship between any available quantitative in vitro susceptibility test and the observed response in vivo to antimicrobial agent therapy. However, these models provide the opportunity for continued evaluation of new test methods and therapeutic strategies as they are envisioned and developed (25a).

Use of Bactericidal Tests for Human Infections

Two strategies have been used in bactericidal testing. In one approach, the concentration of antimicrobial agent needed to kill the organism (MBC) is determined in the laboratory, and the concentration is compared with the concentration of antibiotic actually measured in the body at various time points during a selected dosing interval. The goal of this pharmacokinetic approach is to exceed the MBC of the drug for the infecting microbe in the serum or at the site of infection during all or part of the treatment period. This type of testing is especially cumbersome for the laboratory because drug concentrations as well as MBCs must be determined. In the other approach, serum from a patient who is receiving antimicrobial agent therapy is serially diluted and an inoculum of the infecting organism is added. This test result, rather than being an antimicrobial agent concentration, is a titer that indicates what dilution of the serum is bactericidal to the microbe. While this type of testing appears to be much simpler to perform, the technical and biological variables affecting test performance make interpretation of the test results for a particular patient difficult at best.

Serum dilution (bactericidal titer) test. The serum dilution test is known by several names, including the SBT test and Schlichter test. Evidence for the clinical utility of such tests comes primarily from reports in which the serum dilution test was used to evaluate therapy for bacterial endocarditis (100, 101). As initially described, the serum dilution test measured inhibitory rather than bactericidal activity but was subsequently modified to monitor antimicrobial agent bactericidal activity in serum (31). Fisher (31) introduced the concept of subculturing a portion of the broth culture medium onto antibiotic-free agar to determine whether the original bacterial inoculum was being killed, rather than inhibited, during incubation. The serum dilution test has been recently reviewed (106), and while it will not be discussed extensively here, some comments are required, as the technical variables affecting this test are the same as those associated with MBC testing.

Because the interactions between antimicrobial agents and microorganisms in a living host are complex (3), a brief summary of the clinical applicability of the widely used SBT test is worthwhile. Mellors et al. (71) and Coleman et al. (15) reviewed the value of the SBT test in 17 studies published between 1948 and 1980. Four major variables influenced the outcome of the test results: (i) the time of blood collection, (ii) the size of the bacterial inoculum, (iii) the endpoint definition, and (iv) the diluent used. They were unable to associate patient survival or bacteriological cure with an SBT of $\geq$1:8, one traditional cutoff point used to differentiate appropriate from inappropriate therapy (109). Weinstein et al. reported the results of a prospective study using a microdilution method for the SBT test (116). They suggested that a peak SBT of $\geq$1:32 and a trough titer of $\geq$1:16 were significantly associated with bacteriological cure. Overall, therapy was curative in 93% of 115 study patients, irrespective of the in vitro test results. Even within this carefully planned multicenter study, the test results of the five participating laboratories agreed only within a range of $\pm$2 doubling dilutions for any test sample. This difficulty in establishing test reproducibility was highlighted by Wilson (119), who was unable to show any correlation between tests performed on split patient samples, whether run in separate laboratories or repeated blinded in the same laboratory. In a critical review of the report of Weinstein et al. (116), Mellors et al. (71) considered that, at best, this major, prospective evaluation demonstrated that bactericidal testing provided no useful information for at least 82% of the evaluable patients studied. In addition, the application of bactericidal test results to therapy of infections other than endocarditis has not received rigorous scrutiny but has been simply accepted as being inherently useful (53, 103).

Relationship between SBT and MBC. The MBC (also called the minimum lethal concentration) is designed to be a direct measure of a given antimicrobial agent's ability to exert a bactericidal or killing effect against a selected isolate under defined laboratory conditions. The expectation has been that

the actual MBC of a given agent should be directly related to its SBT, although SBT test results are modified by host serum factors that are invariably present. Jordan and Kawachi (53) reported on a group of 39 patients for whom not only the MBC for the infecting microorganism and antimicrobial agent concentrations in serum were determined, but also the SBT. Thus, the relationship between antimicrobial agent levels, SBT, and clinical outcome could be compared. For the 15 patients receiving a single antimicrobial agent, a low positive correlation ($r = 0.43$) was found between the ratio of the antimicrobial agent concentration in serum to the MBC when plotted against the SBT. Twenty-two sets of data were available for these 15 patients, who were treated initially with one agent for at least 5 days. Thirteen patients had a good outcome, and two died. In 14 patients, trough drug concentrations exceeded the MBC. In nine patients, the peak SBT was ≥1:10, and in six it was lower than this. For one patient who died, only a peak drug (nafcillin) concentration value was available, but it exceeded the MBC by over 20-fold, and the trough SBT was 1:16.

As mentioned earlier in this review, antimicrobial agents bind to serum proteins to various degrees. Since only the free or non-protein-bound drug is biologically active, the degree of protein binding should be taken into account when the SBT test is performed in the presence of serum proteins. For some classes of antibiotics, such as aminoglycosides and fluoroquinolones, the degree of protein binding is negligible. Other antibiotics, such as selected penicillins and cephalosporins, are >90% serum protein bound, and therefore the results may be very different depending on the concentration of serum proteins present during testing. While some laboratories perform SBT tests by diluting the patient's serum in pooled human serum, maintaining a stable serum protein concentration throughout the dilution series, others dilute the patient sample in a broth culture medium so that the serum protein concentration decreases throughout the dilution series. When a highly protein-bound antimicrobial agent is present in the patient's serum, dilution of the sample in serum-enriched medium typically yields a lower SBT (lower bactericidal activity) than does dilution in broth. The MBC test often represents what would be considered free or non-protein-bound antimicrobial activity, whereas in the SBT test, protein may bind and render a portion of the antimicrobial agent biologically inactive. If only free or non-protein-bound drug is considered biologically active (83, 84), then the study by Jordan and Kawachi (53) indicates that the free drug concentrations in the serum exceeded the MBC in 11 patients, including 2 who expired. However, the free drug concentration did not exceed the MBC in four study subjects, all of whom did well clinically. Thus, in this group of patients, quantitative correlation between the MBC and SBT was poor, and neither test predicted therapeutic outcome.

Drusano et al. (20) correlated two methods of MBC testing with SBT data in a controlled experiment with 10 volunteers. They found a correlation between MBC and SBT test results ranging from 48 to 98%, depending on the method used for MBC testing (20). Similarly, Robinson et al. (93) reported on a series of 10 patients for whom poor correlation was found between the SBT (performed to measure the synergistic activity of antimicrobial agent combinations) and other in vitro tests for synergy by the checkerboard technique. These results in humans demonstrate that (i) a quantitative relationship between the measured MBC and the SBT cannot be easily demonstrated and (ii) neither test is accurate in directly predicting the clinical response to antimicrobial therapy.

SUGGESTED CLINICAL USES FOR BACTERICIDAL TESTING

Clinicians often want to use antimicrobial agents that are bactericidal rather than bacteriostatic, and therefore, some type of standard, reproducible MBC analysis is needed. As noted earlier, the administration of bactericidal antimicrobial agents (along with in vitro therapeutic monitoring) has been suggested for treatment of endocarditis (17, 47, 94), sepsis in immunocompromised patients (17, 23, 47, 55, 94), osteomyelitis (47, 111), and other types of chronic infections (47). The use of in vitro monitoring has also been suggested for predicting therapeutic outcome for infectious diseases for which there are no treatment guidelines (27). While the utility of assessing the bactericidal activity of particular drugs for individual patients with these types of infections is controversial, knowing whether an agent or class of agents is considered bactericidal against a particular microorganism(s) may be important. For example, Rahal and Simberkoff (89) showed that chloramphenicol is bactericidal against such meningeal pathogens as *H. influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, whereas it is only bacteriostatic against members of the family *Enterobacteriaceae* and *S. aureus*. The classes of commonly used antimicrobial agents that are considered bactericidal include penicillins, cephalosporins, and related compounds; vancomycin and related compounds; aminoglycosides; quinolones; rifampin; metronidazole; bacitracin; polymyxin; and colistimethate. Those considered bacteriostatic are chloramphenicol; erythromycin, tetracycline, and related compounds; sulfonamides and related agents; and clindamycin (97). Such information can help a clinician select an appropriate antimicrobial agent for patient treatment.

We suggest that routine MBC testing not be offered by clinical microbiology laboratories. There is little evidence that this type of testing is of any benefit in the care of infected patients. A basic understanding of whether the antimicrobial agent(s) being considered for therapy is bacteriostatic or bactericidal is more essential than routine MBC testing. Additional information about the clinical relevance of MBC tests in the management of individual patients is still needed, but this should be gained during well-designed, prospective, blinded clinical studies. The data currently available from retrospective investigations have not proved convincing to many authorities. If testing is performed as an adjunct to ongoing patient care, we strongly recommend that a rigidly standardized procedure be followed, such as that outlined in this report, and that all tests be performed in duplicate with the recommended quality controls.

MBC testing of new antimicrobial agents early in their development is worthwhile to establish whether the new agent generally conforms to the mechanism of antibacterial action consistent with its class. In this testing, too, careful control of procedures is crucial, and tests are likely best performed in laboratories experienced in bactericidal testing.

CONCLUSIONS AND RECOMMENDATIONS

MBC and SBT tests have been performed for decades, yet there is still no consensus as to their clinical utility in direct patient care. Opinions differ on the use of such in vitro susceptibility tests, from those who would eliminate routine

MBC testing (38) to those who consider that lack of standardization should not interfere with the performance and interpretation of MBC tests by clinical microbiologists and their use by clinicians for making decisions about therapy (47).

Following the recommendation of MacLowry (66), we suggest a middle ground. There is a need to evaluate standardized MBC and SBT procedures for their reproducibility and clinical relevance before considering their widespread, direct clinical use. Whenever bactericidal testing is performed, it must be done under the stringent conditions outlined in this article and reference documents (75, 102, 106a). The test should not be performed as part of the routine services offered by the laboratory, but if done, it should be part of a clinical microbiology consultation involving antibiotic treatment for the patient.

The in vitro tests performed in clinical microbiology laboratories need to be standardized, reproducible, and clinically relevant. Clinical laboratory personnel should work toward these goals, involving interested clinicians, and should help to scientifically determine the limitations as well as the benefits of bactericidal testing for seriously infected patients.

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